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A bright and responsive europium probe for determination of pH change within the endoplasmic reticulum of living cells†

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A ratiometric Eu^{III} complex has been developed that localises selectively within the endoplasmic reticulum of living cells. Careful calibration, using a time-gated spectral imaging microscope, allows the intensity ratio of emission bands and the variation of excited state lifetime to be used for pH determination, with a pK_a of 7.15.

The pursuit of non-invasive, responsive optical probes which target specific cellular organelles and signal concentration changes of bioactive species in real time remains a challenging area of research.[1] In particular, probes that can report changes to the pH environment of certain cellular compartments have the potential to be used in the monitoring of several pathological and physiological cellular processes. The critical design factors for these probes require careful consideration. Important factors include rapid cellular uptake, specific organelle localisation, low cytotoxicity, the ability to be calibrated and the retention of probe performance *in cellulo*.

Determination of the pH environment of biochemical events has commonly relied on the use of chromogenic pH indicators which often only take advantage of the lower wavelengths of the electromagnetic spectrum. However, the need for more advanced luminescent systems, which can be employed for more efficient *in vivo* detection in real time, has sparked the development of probes with longer emission lifetimes.[2,3] Such delayed emission has the advantage of overcoming the poor signal-to-noise ratio caused by short lived background emission (autofluorescence) and light scattering from the surrounding biological environment. It is well known that emissive lanthanide complexes are particularly well suited for this purpose, with an extensive variety of examples being reported.[4] Another significant advantage of using lanthanide based probes is their ability to measure fluctuations in the concentration of intracellular species through well established ratiometric methodology. This is most relevant with complexes of europium(III), as the spectral form and emission intensity are highly dependent upon the symmetry and nature of the coordination sphere. In recent work, we have exploited the sensitivity of the $\Delta J = 2$ band to the polarisability of the axial donor, to allow ratiometric analyses of various analytes such as citrate, lactate and bicarbonate.[5,6] The mode of action for these systems involved displacement of a coordinated H_2O molecule, either through direct interaction of the analyte at the metal centre or, in the case of pH monitoring, by reversible pH-dependent intramolecular sulphonamide ligation.

Our previous pH probes have been based on the 1,4,7,10-tetraazacyclododecane macrocyclic framework incorporating either an azathiaxanthone (ε = 6500 M^{-1} cm^{-1}) or azaxanthone (ε = 5700 M^{-1} cm^{-1}) sensitising moiety and exhibiting quantum yields of 6% or less. With this in mind, we have set out to develop an optical pH probe with a higher quantum yield that incorporates multiple sensitising moieties with large molar absorptivity, creating a complex with overall higher brightness, B, where $B(\lambda) = \varepsilon(\lambda)\phi$. A family of Eu^{III} complexes based on triazacyclononane with three *p*-substituted aryl-alkynyl groups to harvest incident light has recently been reported. With quantum yields as high as 50% and molar extinction coefficients in the range of 60 000 M^{-1} cm^{-1}, it was decided to create new pH probes based on such systems.[7] A methyl substituted sulphonamide moiety that can bind reversibly to the lanthanide ion, thereby changing the metal coordination environment, was incorporated into the ligand design (Scheme 1).

The pyridylalkynylaryl based chromophore is known to allow excitation of Eu^{III} over the range 337–365 nm and was prepared using established methods.[7] Subsequent dialkylation of mono-BOC-triazacyclononane, followed by BOC deprotection (TFA-CH_2Cl_2) resulted in the bis-alkylated precursor ligand. Reaction with 2-methanesulfonate-*N*-methanesulfonylethylamine in MeCN in the presence of K_2CO_3 at 70 °C afforded the desired ligand. Prior to complexation with $Eu(OAc)_3$, base hydrolysis of the ethyl phosphinate esters with NaOH in a $CD_3OD : D_2O$ (3 : 1) solution mixture was carried out. Purification by preparative-HPLC gave $[\mathbf{EuL^1}]^+$ in 33% yield.

Examination of the Eu^{III} emission spectra of $[\mathbf{EuL^1}]^+$ as a function of pH (0.1 M NaCl, 298 K) revealed substantial and reversible changes in the fine splitting of the $\Delta J = 1$ transition and also in the form of the $\Delta J = 2$ and $\Delta J = 4$ spectral bands. Upon basification, two new bands at 625 nm and 688 nm were observed. By plotting the change in the emission intensity ratio of selected bands (*e.g.* $\Delta J = 2/[\Delta J = 0 + \Delta J = 1]$) over the pH range 4–9 (Fig. 1) an 80% increase in

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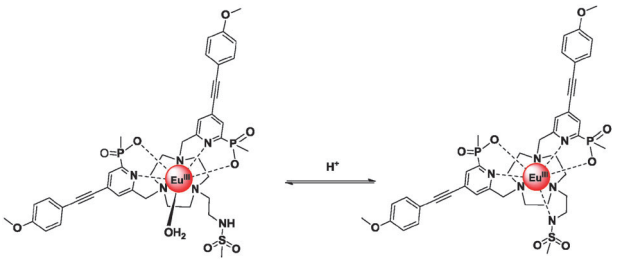

Scheme 1 Representation of pH dependent sulphonamide ligation.

the ratio was found, with a protonation constant of 6.52(±0.05) being estimated. Lifetime measurements for the Eu^{III} excited state of $[\mathbf{EuL^1}]^+$, both in D_2O and H_2O, were recorded at various pH values. At pH 9, a q value of zero was determined with partial hydration being observed under more acidic conditions (q = 0.6 at pH 4). The more intense emission observed at higher pH is characterised by an extremely high overall emission quantum yield of 38%. At pH 6.5, the quantum yield reduced to 16%.

In order to gain insight into the response of $[\mathbf{EuL^1}]^+$ *in cellulo*, its emissive behaviour was examined in a 'simulated extracellular' ionic background consisting of 0.9 mM hydrogen phosphate, 0.13 mM citrate, 2.3 mM lactate, 30 mM bicarbonate, 0.1 M NaCl and 0.4 mM human serum albumin (HSA). A shift in the protonation constant associated with reversible intramolecular sulphonamide ligation to 7.10(±0.03) was observed, owing to a competitive protein interaction with the rod-like alkynyl sensitising moieties and anion chelation at the Eu^{III} metal centre. Furthermore, the Eu^{III} emission intensity showed an overall increase upon acidification. Confirmation of complex interference within this competitive medium was gained by comparing the spectral form of $[\mathbf{EuL^1}]^+$ at pH 9 to that recorded in the absence of any protein or anions. Significant changes in the fine splitting of the ΔJ = 1, ΔJ = 2 and ΔJ = 4 transitions were observed, consistent with a change in the Eu^{III} coordination environment.

Significant competition to intramolecular sulphonamide ligation comes from intermolecular HCO_3^- binding and this was hypothesised as the principle cause of the spectral changes. This aspect was investigated by recording an emission spectrum of $[\mathbf{EuL^1}]^+$ in the presence of 30 mM HCO_3^- alone. The resulting spectral form was identical to that observed when the mixed 'simulated extracellular' media was used. For each of the other biologically relevant anions, no

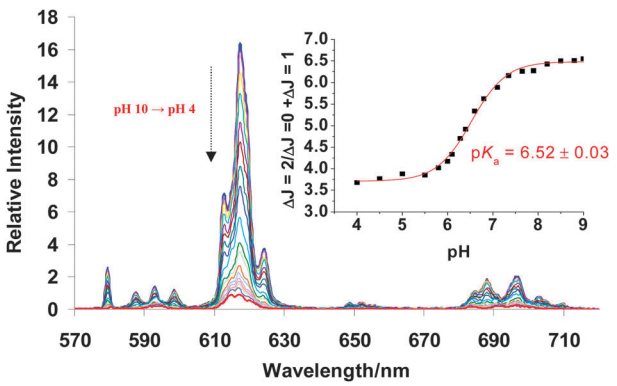

Fig. 1 Variation of the EuIII emission of $[\mathbf{EuL^1}]^+$ as a function of pH (H_2O, 5 μM complex, 298 K, I = 0.1 M NaCl, λ_{exc} = 332 nm). Inset: plot of $\Delta J = 2/(\Delta J = 0 + \Delta J = 1)$ *versus* pH, showing the fit to the observed data for an apparent pK_a = 6.52(±0.03).

Table 1 Rate constants k^b (ms^{-1} ±10%) for depopulation of the Eu^{III} $^5D_0$ excited state and hydration state q^a, (±0.2) for $[\mathbf{EuL^1}]^+$ in the presence of 0.1 M NaCl or 0.4 mM HSA at varying pH values (λ_{exc} = 332 nm, 298 K)

$[\mathbf{EuL^1}]$	pH	$k(H_2O)$	$k(D_2O)$	q^{Eu}
0.1 M NaCl	4.5	1.54	0.80	0.6
	7.0	1.72	1.34	0.2
	9.5	1.75	1.46	0
0.4 mM HSA[c]	4.5	0.99	0.72	0
	9.5	1.26	1.23	0

[a] q values were estimated according to ref. 8 and assume fast NH/DH exchange for the unbound sulphonamide. [b] Values represent the mean of three separate measurements. [c] At pH 9, with excess bicarbonate in H_2O and D_2O, k values were 1.33 and 1.05 ms^{-1} respectively, q = 0.04.

significant changes were observed in the profile or relative intensities of the Eu^{III} emission bands. In relation to protein interference, at pH 9 no major structural changes in the Eu^{III} emission were observed, so that the concept of direct binding of the protein functional groups to the Eu^{III} centre and thus displacement of the sulphonamide moiety can be eliminated. As expected, upon acidification (pH 4), a different spectral form to that seen in the absence of HSA was observed, suggesting possible protein binding to the metal centre at lower pH. Excited state lifetime measurements also confirmed that in both acidic (pH 4) and basic (pH 9) media, $[\mathbf{EuL^1}]^+$ had a hydration state of zero (Table 1).

Recently, a structurally similar complex to $[\mathbf{EuL^1}]^+$, with three methoxy pyridylalkynylaryl chromophore units exhibited selective staining of the mitochondria in a variety of cell lines (NIH-3T3, CHO and PC-3).[7] However, it was not known whether replacement of one of these antenna moieties with the methyl sulphonamide pendant arm would have a significant effect on the localisation profile. Indeed, cellular localisation studies with $[\mathbf{EuL^1}]^+$, using a concentration of 30 μM and incubation times of between 30 min and 48 h showed little staining of the mitochondrial region of CHO and NIH-3T3 cells. Nevertheless, co-staining experiments with ER Tracker Green confirmed significant uptake in the endoplasmic reticulum with 90(±2)% correspondence being determined (Fig. 2).

Incubation times of up to 24 h were investigated, but little change in the intensity of the staining was observed after 4 h, suggesting relatively quick localisation within the ER(±5%). Cytotoxicity studies using an MTT assay determined an IC_{50} value of greater than 100 μM for $[\mathbf{EuL^1}]^+$, over a 24 h period,[9] and ICP-MS measurements of 4 × 10^6 NIH 3T3 cells loaded with 30 μM $[\mathbf{EuL^1}]$ for 24 h at pH 5.6 indicated that the total intracellular concentration was 390 μM. Interestingly, at higher pH (7.7), a decrease of *ca.* 50% in intracellular complex concentration was measured, suggesting uptake and accumulation processes for $[\mathbf{EuL^1}]^+$ may occur more efficiently at lower pH.

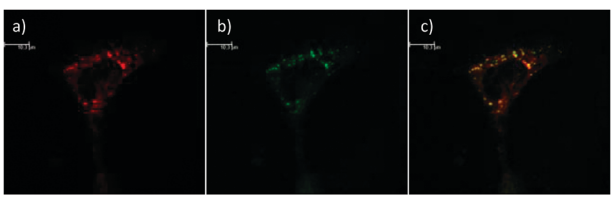

Fig. 2 (a) LSCM microscopy images of NIH 3T3 cells (mouse skin fibroblasts) loaded with $[\mathbf{EuL^1}]^+$ (4 h incubation, 30 μM complex concentration in the growth medium); λ_{exc} = 355 nm, λ_{em} = 605–720 nm (b) corresponding ER Tracker Green emission (λ_{exc} = 496 nm, λ_{em} = 505–530 nm) (c) RGB merge image (P = 0.90).

Previous studies by Kim *et al.*[10] demonstrated that due to the large proton permeability of the ER membrane, significant communication occurs with the homeostatic mechanism that controls cytosolic pH. Thus, the value of pH_{ER} is similar to that found within the cytoplasm (7.1). Therefore, any knowledge gained on pH changes of the ER region using $[\mathbf{EuL^1}]^+$, provides information on overall cytosolic pH. In order to ensure the probe retains its ability to respond to pH changes *in cellulo*, a preliminary experiment was carried out where the pH_{ER} was altered from 7.7 to 6.0 (by varying the pH of the growth medium and adding nigericin (0.2 μM) to allow K^+/H^+ exchange) and the response of $[\mathbf{EuL^1}]^+$ determined by microscopy. By changing the pH of the cell culture media from 7.7 to 6.0, a two fold increase in brightness within the ER organelles was observed. Using the Leica SP5 II LSCM microscope equipped with a high sensitivity hybrid detector (HyD), changes in the emission intensities of the various individual selected Eu^{III} transitions were monitored. This involved extracting an average intensity value *via* a contrast transfer function (CTF) for each band, from 15 small (250 × 250 pixel) voxels (pixel size 120 nm × 120 nm × 0.772 μm) within the image which represented the stained ER regions of the cell. The largest change in emission band ratio with pH was observed for $\Delta J = 2/\Delta J = 4$, where the ratio increased from 1.8 at pH 7.7 to 2.2 at pH 6.0.

A more detailed *in cellulo* calibration experiment was carried out using a custom-built, time-gated spectral imaging microscope, where spectra and excited state lifetimes for $[\mathbf{EuL^1}]^+$ within the ER regions of the cell (4 h incubation) were recorded at 11 different pH points between pH 7.5 and 6.0. An experimental technique was employed using growth media with different pH values, along with the addition of nigericin (0.2 μM) at each loading to ensure pH_{ER} equilibration (ESI†). To ensure that any changes in pCO_2 within the incubation chamber did not alter the pH of the growth medium, it was necessary before loading to allow equilibration of the partial pressure of CO_2 in the solutions and re-adjust the pH (×3). As observed for the *in vitro* based pH titration carried out in the 'simulated extracellular' ionic background, acidification led to an overall increase in emission intensity along with changes in spectral form. By plotting $\Delta J = 2/(\Delta J = 0 + \Delta J = 1)$ against pH, it was apparent that a near linear response was observed between the pH range 6.7 and 7.4, with an overall 40% change in ratio measured within this region. Changes in the excited state lifetimes were also observed, with values of 672 μs and 413 μs recorded at pH 6.7 and pH 7.4, respectively. This 60% increase in the lifetime upon lowering the pH within the ER, suggests that changes in the structural form of the complex when the sulphonamide moiety is unbound may be affecting certain protein–complex interactions, resulting in longer lifetime values due to a reduction in the quenching process. Comparing the spectral intensity ratio changes with the pH/lifetime variation (Fig. 3), a crossover point occurs at pH 7.15(±0.05), in agreement with the pK_a value (7.10(±0.03)), obtained for $[\mathbf{EuL^1}]$ in a competitive anion/protein environment.

In summary, using $[\mathbf{EuL^1}]^+$, this calibration procedure provides an efficient method for determining pH change within the endoplasmic reticulum of living mammalian cells, either through ratiometric comparison of two emission bands or by measurement of its excited state lifetime. Each method is independent of complex concentration. It is believed that the ER pH mimics that found within the cytoplasm, so that this probe also provides information on mean cytosolic pH. Such behaviour augurs well for the development of related responsive systems (*e.g.* pM, pX) that can faithfully report concentration changes in particular cell compartments of living cells.

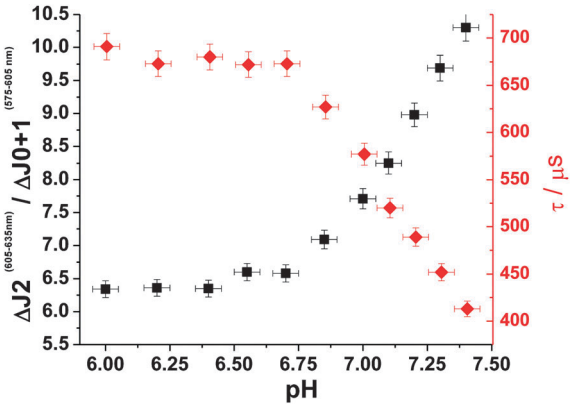

Fig. 3 Changes in the Eu emission intensity ratio $\Delta J = 2/(\Delta J = 0 + \Delta J = 1)$ and excited state lifetimes (τ) for $[\mathbf{EuL^1}]^+$ as a f(pH).

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